

## Fractionation of Gelatins with Carboxymethyl Cellulose

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Commercial gelatins could be divided into 2 fractions using carboxymethyl cellulose columns which were eluted with various salt strength gradients. The pattern varied according to the history of the gelatin preparation. Analogous experiments with carboxymethyl cellulose papers are also described.

We have earlier described fractionations of commercial gelatins with a cation exchange resin<sup>1</sup> and wished to confirm the findings with a corresponding cation exchange cellulose. Preliminary experiments were carried out on CM-cellulose paper but we could not apply directly the conditions of exchange resin fractionation. It was found instead that with salt solutions the gelatins could be eluted in fractions from the CM-cellulose and this finding was applied to columns. This final phase of the work is related to the experiments of Kessler, Rosen and Levenson<sup>2</sup>, who studied acetic acid-solubilized rat tail tendon collagens, and to the work by Piez, Weiss and Lewis<sup>3</sup> on mildly denatured skin collagens.

### EXPERIMENTAL

**Materials.** The carboxymethyl celluloses (referred to here as CM-celluloses) were Whatman products, purchased from H. Reeve Angel & Co. Ltd., London. The powder was labelled "Cation Exchanger Carboxymethylcellulose Powder CM 70" and supplied in sodium form. Two experimental batches of paper (designated CM 50) were used. Even if their ion-binding capacity was reportedly the same, they differed considerably (see Fig. 4). At present the reason for this variation is unknown to us. The manufacturers also informed us that "... there are subtle differences in the behaviour of different batches ...."

The gelatin samples I, II and IV were obtained from commercial sources, III was a laboratory preparation according to Jackson<sup>4</sup>, V—IX were specially prepared by Kind & Knox Gelatin Company (Camden, New Jersey, U.S.A.). The preparations III—IX are the same as those used earlier<sup>1</sup> by us. The present designations I and II correspond to II and I, respectively. The pI was determined as the pH of the solution which had passed through a "mixed-bed resin" column<sup>5</sup>.

- I. clear, strong jelly, prepared from "cartilage and good bones", pI 5.2;
- II. turbid (clear when heated), poor jelly, pI 5.3;
- III. laboratory preparation, turbid even when heated, good jelly, pI 6.9;
- IV. prepared for bacteriological purposes, strong jelly, pI 7.5;
- V. specially prepared from pork skin after acidic pretreatment, Bloom value 304, pI 6.8;

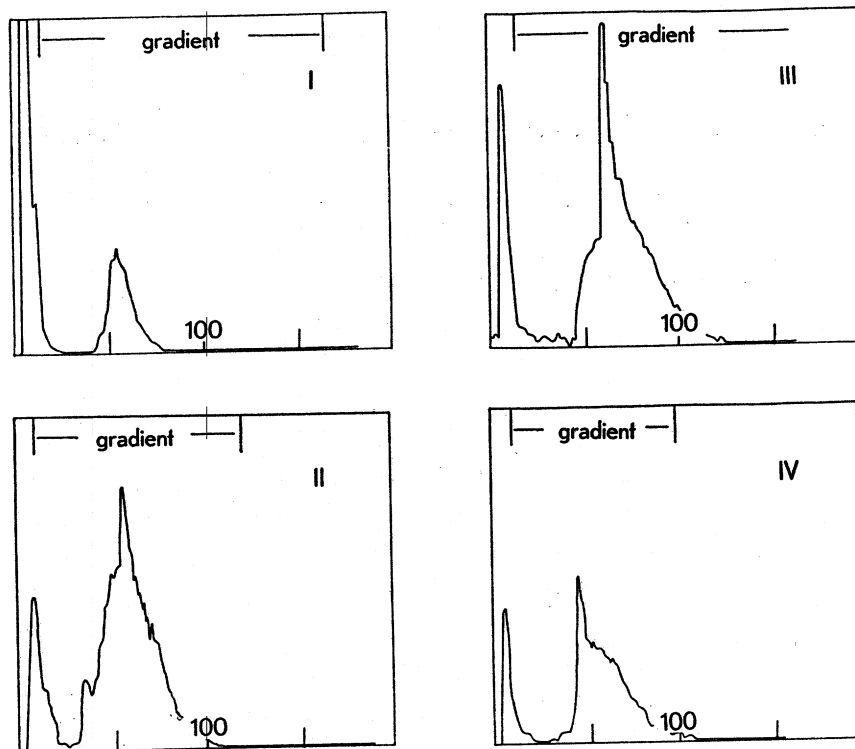


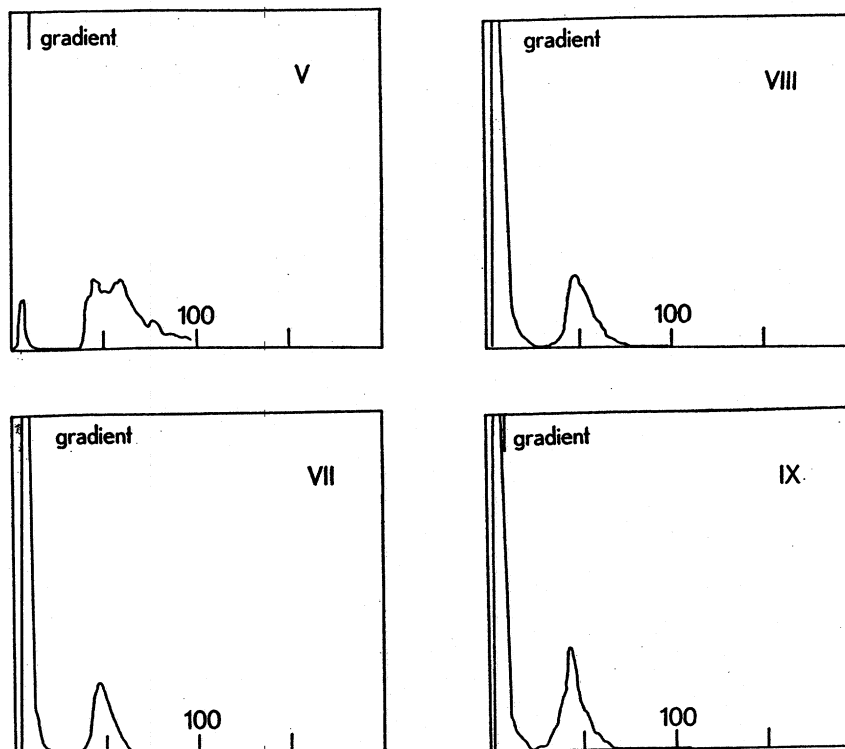
Fig. 1. Fractionation pattern of several samples of gelatins (indicated by numbers I—IX, specified in the text; VIA in Fig. 3 is also comparable) using carboxymethyl cellulose columns. The beginning of the salt gradient and the point when 100 ml eluate had emerged

- VI. second run after V; Bloom value 172, pI 6.8;
- VII. specially prepared from limed calf skin, Bloom value 244, pI 4.95;
- VIII. second run after VII, Bloom value 218, pI 4.9;
- IX. third run after VII—VIII, Bloom value 124, pI 4.85.

*Fractionation with CM-cellulose columns.* The CM-cellulose powder (in sodium form) was suspended in water and allowed to swell overnight. It was washed by decantation and the fine parts were thus removed. The suspension was left to settle in a 1 cm diameter water-jacketed tube in a column of 15 cm height. Finally, air pressure (1 atm) was used to pack the column. A temperature of 37°C was maintained in the column as a rule, but at room temperature the fractionation pattern was almost similar.

If the column was treated with dilute (0.01 M) acetate buffers before the application of the samples, the distribution of the fractions was greatly affected and the significance of pH will be described later.

The gelatin sample (about 40 mg as a 1 % solution which had been passed through a "mixed-bed resin" to remove the ions) was warmed to 40°C and allowed to drain into the column. It was eluted first with water and then with salt solution. Most of the earlier experiments were made using magnesium chloride solution as eluent. The feeding vessel (stirred magnetically) contained initially 100 ml water, and 0.7 %  $MgCl_2$  was employed to replace the eluent used from the reservoir flask. Latter, an equivalent concentration of sodium chloride was used. One ml fractions were taken using a "drop-counting" fraction collector.



are indicated. At the "100 ml point", the concentration of the salt was about 0.092 N. The total ordinate corresponds to an extinction of 0.9. The analyses are carried out of 0.5 ml eluate, except of 0.3 ml in fractionation of sample V.

Several other salts were tried in equivalent concentrations: potassium chloride, sodium chloride, lithium chloride, calcium chloride, zinc chloride and potassium thiocyanate. However, they did not give markedly different results. Also, the effects of the presence of urea and of pretreatment with hydroxylamine were investigated.

**Fractionation on CM-cellulose paper.** Two cm broad strips were placed in chromatography troughs for conventional descending elution. A saturation of the paper with water vapour was not necessary. Usually the experiment was carried out at room temperature since at 37°C no improvement was obtained. The gelatin sample (0.010–0.050 ml 1 % solution, heated for 10 min at 60–70°C) was pipetted on the starting line which had been previously moistened with water. After irrigation with the desired solution, the strip was dried either at room temperature or at 100°C and stained as described below. Additional chromatograms were obtained on washed papers as described in the 'results' section. The difference between the two batches of "CM 50" paper persisted also on the washed paper.

**Localization of the fractions.** The fractions obtained from the CM-cellulose column were located using a modification of the biuret reaction introduced by Lowry and co-workers<sup>6</sup>. If a precipitate of calcium, magnesium or zinc hydroxide was formed, the fluids were centrifuged clear before the measurement of the colour. In one sample of collagen (V), the magnesium chloride disturbed the development of the colour.

It was necessary to find a satisfactory method for staining the gelatin also on the paper. The following procedure was finally adopted: The dried papers were dipped for

5 min into 1 % tetrabromophenolsulphonphthalein solution<sup>7</sup> (in ethanol and saturated with  $\text{HgCl}_2$ ). After washing in 0.5 % acetic acid solution (3 times for 10 min), the papers were dried between blotting papers. Washing with ethanol or methanol did not produce any better contrast. In ammonia vapor, the yellow stain turned blue and distinct but the spots became very soluble. Some other procedures were also tried: spraying with ninhydrin solution (suitable but not very sensitive), collagen staining with aniline blue after treatment with phosphotungstic acid<sup>8</sup> (complicated and not sensitive enough) and staining with lissamine green and subsequent washing with 3 % acetic acid<sup>9</sup> (suitable but not sensitive).

*Physicochemical determinations.* Sedimentation was measured with a Spinco analytical ultracentrifuge Model E, diffusion with Antweiler's microelectrophoresis apparatus using the diffusion cell and viscosity with an Ostwald type viscosimeter.

## RESULTS

*Fractionation with CM-cellulose columns.* Two fractions were always obtained (Fig. 1). The first fraction seemingly was not attached to the CM-cellulose at all. However, when the CM-cellulose was in the hydrogen form, even the first fraction was bound to the CM-cellulose and was eluted with very dilute magnesium chloride solution. Thus the small amount of sodium in the CM-cellulose was enough to prevent the association of this fraction to the CM-cellulose. The acid gelatins (from limed precursors) yielded a large first fraction which passed straight through the column. We do not give any quantitative estimations concerning the proportions of these fractions since the biuret reaction is approximate only and the amount in the tubes may slightly fluctuate. Fig. 2 shows that the fractions can be reproducibly rechromatographed. The fractionation in the presence of urea did not give markedly different results. A pretreatment with hydroxylamine (pH 9, concentration of hydroxylamine 0.5 M, 90 min at 40°C) of sample V reduced the first fraction.

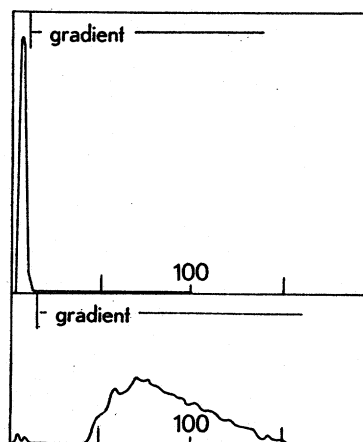
When some samples got infected and did not form gel any more, the fractionation pattern changed and new fractions appeared (Fig. 3).

*Fractionation on CM-cellulose paper.* The initial experiments with CM-cellulose paper were made with pH 5.5 citric acid-sodium phosphate buffer (McIlwaine) as eluent. It was observed that a part of the sample (about half) was retained at the origin, the other part migrating with an  $R_F$  of about 0.4–0.5. On a paper which had been prewashed with this buffer, the whole sample moved with the front. When a strip was eluted first with pH 5.5 buffer and then with sodium hydroxide, the whole material also migrated at the front. When a strip was developed with the pH 5.5 buffer and sprayed with a suitable pH indicator, it was found that the McIlwaine's buffer was fractionated by "frontal analysis" during the run and a part of the gelatin migrated on the pH boundary line. This conclusion was supported by the finding that, with a pH 8.6 barbiturate buffer, a similar fractionation was obtained.

In addition, 3 % ammonia and 0.1 N hydrochloric acid were tried as eluents but proved unsuccessful. At 4°C, that fraction which was retained at the origin was much augmented.

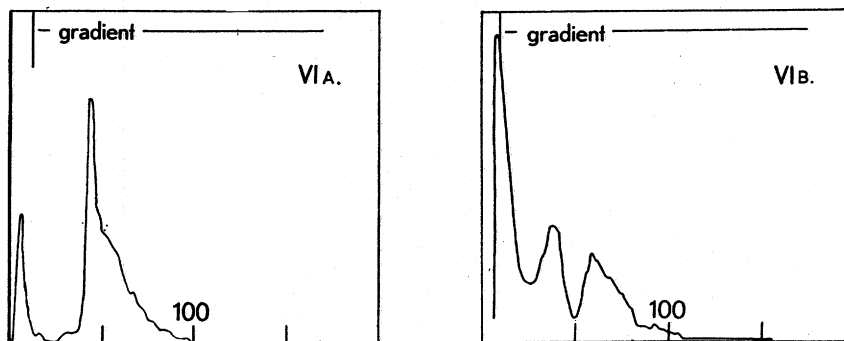
The best separations were obtained with 0.7 %  $\text{MgCl}_2$  using the first batch of CM-cellulose paper (Fig. 4). Five bands were registered with  $R_F$  values of zero (weak), 0.55 (weak), 0.83, 0.87 (weak) and 1.00 (sample III). From Merck's gelatin (I), only the fraction with  $R_F = 0.83$  was obtained.

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*Fig. 2.* Rechromatography of the fractions:  
Above: First fraction shown in Fig. 1:VII.  
Below: Second fraction shown in Fig. 1:III.

To compare different salts, an experiment was made on the second batch of paper at  $+37^{\circ}\text{C}$  with  $\text{KCl}$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{KSCN}$ ,  $\text{NaCl}$  and  $\text{CaCl}_2$ . The molar concentrations of the eluting fluids were adjusted to correspond to 0.3 %  $\text{MgCl}_2$ . Sodium chloride, potassium chloride and potassium thiocyanate were ineffective and the material remained at the starting line. (In the column fractionation, potassium and sodium chlorides were comparable to  $\text{MgCl}_2$ .) Calcium chloride gave a similar result to  $\text{MgCl}_2$ . Sodium sulphate eluted a part with the front and part was retained at the origin. Similar attempts were made with acetate buffer, pH 4.8. With 0.1 M buffer, the bulk of all the samples migrated with the front. The strength 0.05–0.07 M was found suitable for fractionation.



*Fig. 3.* Effect of microbial degradation on the fractionation pattern. VIA original (without buffer), VIB infected sample (the column pretreated with pH 4.8, 0.01 M acetate buffer). When the original sample VIA was chromatographed in the presence of buffer, only the main peak appeared at about "40 ml point", but no forepeak.

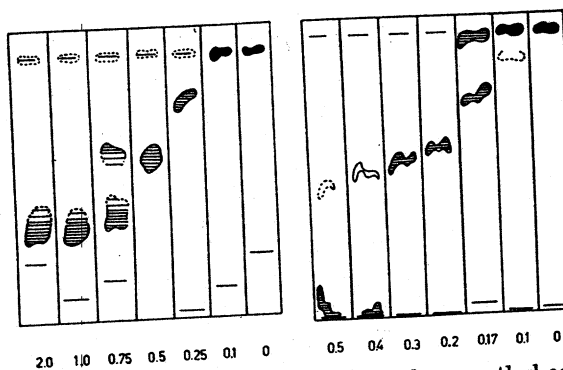


Fig. 4. Elution chromatography of gelatin (III) on carboxymethyl cellulose paper strips. The origin on the top, the solvent front marked below. The figures give the concentration (in %) of magnesium chloride which had been used in the elution. The staining was performed with bromophenolblue<sup>7</sup>. The difference in the two batches of paper is evident.

Most gelatins were then divided into two fractions, one retained at the origin, the other migrating with an  $R_F$  of about 0.5. There were differences between the various samples. Sample I (pI 5.2) was retained least under all the conditions studied. An attempt was also made with  $MgCl_2$  elution on paper that had been washed with pH 4.8 buffer. As expected, the  $R_F$  value of the slower-moving fractions was decreased at pH 4.8 indicating a stronger binding on the CM-cellulose.

**Physicochemical data.** The two fractions of sample I were studied. The sedimentation coefficients ( $s_{20,w}$ ) were 2.95  $S$  and 2.29  $S$ . The intrinsic viscosities were 0.39 and 0.25 ( $c = g/100\text{ ml}$ ), respectively. The diffusion coefficient of the first fraction was  $D_{20,w} = 3.9 \times 10^{-7} \text{ cm}^2/\text{sec}$ . All measurements were made in pH 4.8, 0.1 M acetate buffer at 37°C. The concentrations were based on the biuret reaction.

#### DISCUSSION

At present, further experiments on carboxymethyl cellulose paper do not seem advisable because of the heterogeneity of the commercial CM-cellulose papers. Even if the capacity to bind sodium ions is the same, other unknown properties seem to affect significantly the binding of gelatin. However, when this property of the papers becomes understood, methods can be developed for the rapid screening of gelatins.

On the basis of experiments at various hydrogen ion concentrations we believe that the present procedure is related to the fractionation with Amberlite CG-50 resin<sup>1</sup>. The latter is operated with higher salt strengths which affect the apparent isoelectric points of the gelatins and the fractions cannot be directly compared.

We believe that the "forepeak" described by Kessler *et al.*<sup>2</sup> would contain far-degraded gelatins and because we have operated with lower salt strengths, we have actually extended their method to smaller fragments of collagen molecule and divided their "forepeak" to its subfractions. The fractions obtained

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by Piez *et al.*<sup>3,10</sup> with sodium acetate buffer gradient from gelatinized soluble collagens can also be separated with the present procedure. Experiments are in progress to elucidate whether the breakdown of these large fragments of collagen can be traced to smaller fractions.

The experience with infected gelatins (Fig. 3) suggests that the present procedure could be applied for the study of the relation of gel-forming capacity and structure of gelatins, for checking their purity and, generally, to follow the degradation of collagens for structural studies. It is useful also for the purification of gelatinized collagen, *e.g.* for the measurement of radioactivity of collagen in metabolic experiments.

*Acknowledgement.* This work is a part of a program supported by a grant from the U. S. Department of Agriculture. We are especially indebted to Dr. J. Naghski, Eastern Utilization Research and Development Division, Agricultural Research Service of U.S. Department of Agriculture, who arranged the supply of the samples V—IX from Kind & Knox Gelatin Company, Camden, N. J., U.S.A., through the courtesy of Dr. E. M. Marks. We also thank Dr. Pentti Seppälä for advice on the determinations of the physicochemical constants.

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